Short Communication

A DNA prime, orally delivered protein boost vaccination strategy against viral encephalitis

Mark P Zanin,[1] Diane E Webster,[1,2] and Steven L Wesselingh[1,3]

[1] The Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia; [2] School of Biological Sciences and Department of Immunology; and [3] Department of Medicine, Monash University, Melbourne, Victoria, Australia

Arboviruses of the families *Togaviridae* and *Flaviviridae* are widely distributed and are important causative agents of viral encephalitis, a severe and often fatal disease. The only internationally available vaccine against these diseases is expensive and laborious to manufacture and difficult to administer. Therefore, new vaccines are required against these pathogens. This study investigates the use of a DNA-prime, orally delivered protein boost vaccination strategy against viral encephalitis. This vaccination strategy was immunogenic and provided partial protection against viral encephalitis in a murine model, demonstrating the possible applicability of this vaccination strategy for the management of endemic encephalitis. *Journal of NeuroVirology* (2007) **13,** 284–289.

Keywords: arbovirus; DNA; encephalitis; mucosal; oral; prime-boost; Sindbis virus; vaccination

Arboviruses that cause encephalitis in humans are widely distributed and place a significant disease burden on affected communities. Viral encephalitis is a severe disease that results in the damage of large areas of the central nervous system (CNS) due to virally induced neuronal death and the inflammatory immune response (Jan and Griffin, 1999; Nargi-Aizenman and Griffin, 2001). Case-fatality rates vary depending on the causative virus but range from 3% to 70% for viruses of the families *Flaviviridae* and *Togaviridae* (reviewed in Tsai *et al*, 1999). The most medically important arbovirus is the Japanese encephalitis (JE) virus, which is responsible for 50,000 cases of encephalitis annually, 10,000 of which are fatal (World health Organization [WHO], 2006). The JE virus is distributed throughout Asia and is spreading beyond its traditional boundaries to new parts of India and, as of 1998, Australia (Hanna *et al*, 1996, 1999; Igarashi *et al*, 1994). The only vaccine available internationally against JE is a mouse brain-derived inactivated (MBDI) vaccine, although cell culture–derived inactivated and –attenuated vaccines are in use in the People's Republic of China (WHO, 2006). Vaccines against other arboviral encephalitides are not generally available.

The MBDI vaccine against JE is difficult to administer in resource-poor regions owing to a reliance on injection, which requires equipment and trained medical personnel and carries the risk of transmission of blood-borne pathogens if performed incorrectly. The requirement for several primary and booster doses and the reliance on a cold chain for distribution further hinders vaccine coverage (reviewed in Tsai *et al*, 1999; WHO, 2006). The effective distribution and administration of this vaccine is consequently limited in many of the resource poor regions where JE is endemic. Therefore, the development of new vaccines is a priority.

DNA vaccines are heat stable, comparatively inexpensive, and easy to produce using bacterial culture and plasmid preparation protocols (Chen *et al*, 1999; Deliyannis *et al*, 2000). DNA vaccines are also

Prime-boost vaccination against viral encephalitis
MP Zanin *et al*
285

relatively safe as they are free of extraneous vaccine components that can cause unwanted reactions, which have been observed during the use of the MBDI vaccine (Berg *et al*, 1997; Chen *et al*, 1999; Deliyannis *et al*, 2000). Whereas the DNA vaccines studied here are reliant on injection, oral administration of DNA vaccines has been performed successfully in several studies against a variety of pathogens (Zheng *et al*, 2002). Vaccination via the mucosal route also has the potential to eliminate many of the problems associated with current vaccines against JE. The comparative ease of mucosal delivery, especially via oral inoculation, means that delivery is noninvasive and less training is required to administer the vaccine. The use of subunits rather than whole pathogens is an added safety feature as it eliminates the risk of reversion to virulence, which exists with the use of attenuated vaccines.

This report details the early development of a DNA-prime, orally delivered protein boost vaccination strategy against viral encephalitis aimed at overcoming the shortcomings of the current vaccine. The murine model of Sindbis virus (SV)-induced encephalitis was used to assess vaccine efficacy. SV-induced encephalitis in mice is a very similar disease to arboviral-induced encephalitis in humans, making this a good model with which to study these diseases (Griffin and Johnson, 1977). The major immunological target of SV is the E2 protein, a constituent of the SV glycoprotein spike complex, which contains the major B cell epitopes of SV (Schoepp and Johnston, 1993). The DNA vaccines and protein antigen consisted of the *E2* gene or the E2 protein, respectively.

DNA vaccines used in this study were based on the pCI-neo vector (Promega) containing the SV *E2* gene with an upstream Kozak sequence and a downstream FLAG tag (pNF). The capacity of these constructs to serve as a template for protein translation *in vitro* was demonstrated by expression of the E2 protein in a cell-free rabbit reticulocyte lysate system (Promega) and in transfected mammalian COS-1 cells (Figure 1). These experiments showed that the plasmids were functional in mammalian cells. Preliminary mouse experiments also showed that these constructs were capable of eliciting humoral and cellular immune responses when injected intramuscularly (data not shown). The protein-based subunit vaccine was expressed in *Esherichia coli* BL21(DE3) as a SV E2–maltose-binding protein fusion (MBP-E2), to facilitate solubility and purification. The expression and purification of MBP-E2 yielded a mixture consisting of proteins of the expected sizes of MBP-E2 and MBP fused to fragments of E2 (Figure 2). A preliminary mouse experiment demonstrated that MBP-E2 was immunogenic via intraperitoneal injection and the resulting humoral immune response provided partial protection against challenge (data not shown).

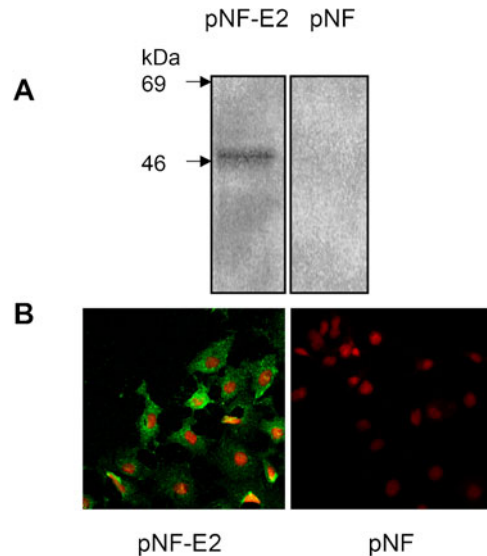These vaccines were then combined in a DNA-prime, oral protein boost vaccination strategy. Two

Figure 1 Characterization and detection of proteins expressed by DNA vaccine constructs *in vitro* and in mammalian cells. (**A**) DNA constructs pCI-neo-FLAG (pNF)-E2 or pNF were added to a rabbit reticulocyte lysate and T7 polymerase mixture and proteins expressed were metabolically labelled with ^{35}*S*-methionine. (**B**) COS-1 cells were transfected with DNA constructs pNF-E2 or pNF. Cells were stained with propidium iodide and probed with anti-FLAG fluoroscein isothiocyanate (FITC)-conjugated antibodies. Cells were observed by confocal microscopy and images were recorded using FITC and Texas Red filters. The data shown are representative of two independent experiments.

doses of 50 μg of DNA were administered intramuscularly on days 0 and 14 followed by doses of recombinant MBP-E2 protein or saline, administered orally, on days 21, 28, and 35. Doses of protein and saline contained 2.5 mg of crude saponins (Sigma), which has been shown to be an effective oral adjuvant (Maharaj *et al*, 1986; Pickering *et al*, 2006). A positive-control group received two doses of 2500 plaque-forming units of the attenuated 633 strain of Sindbis virus intraperitoneally on days 0 and 14 and were not boosted (IP633). All mice were challenged
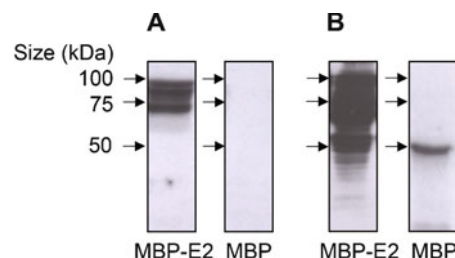

Figure 2 Soluble expression and purification of maltose-binding protein–E2 fusions. Bacterial cultures were induced to express protein and incubated at 18°C for 3 h prior to lysis and purification of MBP-E2 using amylose binding resin. Purified proteins (2.5 μg) were electrophoresed using SDS-PAGE and (**A**) Western blotted and probed with hyperimmune mouse serum against Sindbis virus or (**B**) Western blotted and probed with anti–maltose-binding protein antibodies. The data shown are representative of five independent experiments.

intranasally on day 49 with 2000 plaque-forming units of neuroadapted SV, a virulent, murine adapted strain of SV (Dropulic *et al*, 1997). This challenge consistently causes overwhelming encephalitis in mice up to 12 weeks of age (data not shown).

Each group consisted of 10 3-week-old female BALB/c mice and the groups were organized as follows: four mice were killed on day 55 to obtain serum samples, spleens and brains. The remaining six mice were not killed until the end of the experiment on day 71 unless they displayed symptoms of encephalitis. Serum samples, spleens, and brains were also taken from mice when killed. Serum samples were assayed for anti-SV immunoglobulin G (IgG), IgG1, and IgG2a via enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with concentrated SV particles. Splenocytes isolated from removed spleens were stimulated with inactivated SV strain 633 for 3 days and the culture medium was then removed and assayed for the presence of interferon gamma (IFN-γ) via a sandwich ELISA (Progen). Brain tissue removed from mice was homogenized in enriched Eagle's Basal cell culture medium and the resulting suspension was assayed for the presence of SV in a plaque assay using confluent monolayers of baby hamster kidney cells.

The prime boost vaccination strategy was effective in delaying the development of encephalitis in vaccinated mice. This was particularly evident 7 days post challenge, when all unvaccinated mice displayed symptoms of encephalitis and were killed whereas 50% of vaccinated mice were still protected, showing only mild or no symptoms of encephalitis. Greater mean titers of anti-SV serum IgG antibodies were detected in vaccinated mice than in unvaccinated mice and these differences were statistically significant ($P < .05$) (Figure 3A). The importance of humoral immunity is supported by published literature (Levine *et al*, 1991; Ubol *et al*, 1995).

Cellular immune responses measured 5 days post challenge were greater in vaccinated mice than in unvaccinated mice (Figure 3B). However, at death, the cellular immune response had decreased in vaccinated mice and increased in unvaccinated mice such that, with the exception of the IP633 group, unvaccinated mice had greater mean IFN-γ titers than vaccinated mice, although statistically significant differences were not observed (Figure 3B). These data suggest that the immune response to challenge was skewed to cellular immunity in unvaccinated mice and towards humoral immunity in vaccinated mice. This is supported by analysis of the mean anti-SV serum IgG1:IgG2a titer ratios. The production of IgG1 is indicative of a Th2-type response whereas the production of IgG2a is indicative of a Th1-type response (Figure 3C). These data show that the immune responses elicited by challenge were skewed toward a humoral immune response in vaccinated mice and toward a cellular immune response in unvaccinated mice (Figure 3C).

Measurement of mean viral loads in the CNS indicated that vaccinated mice were better able to clear virus from their brains than unvaccinated mice, which may have limited virally induced pathology in the CNS of vaccinated mice. This was evident of vaccinated mice having lower mean viral loads in their CNS at death than unvaccinated mice (*P* values of .0409, .0211, and .0885 comparing pNF-saline viral loads to those of pNF-E2/E2, pNF-E2/saline, and pNF/E2 at death, respectively) (Figure 3D). The decrease in viral load from 5 days post challenge to death was also greater in vaccinated mice than in unvaccinated mice, indicating the rate of viral clearance in the CNS of vaccinated mice was greater than in unvaccinated mice (Figure 3D). The decrease in viral loads in the CNS from 5 days post challenge to death also demonstrates that mice did not die at the peak of viral replication in the CNS, as shown in other studies (Figure 3D) (Griffin, 1981).

Pearson correlations were performed to determine if there was a relationship between any of the variables measured. A strong negative correlation was observed between viral load and length of survival (Rho $= -0.815$, $P < .0001$, $n = 64$), indicating that viral load in the brain provided a sub-protective measure of vaccine efficacy. Correlations were also observed between anti-SV serum IgG titers and extended survival (Rho $= 0.474$, $P = .0003$, $n = 53$) and anti-SV serum IgG titers and viral load (Rho $= -0.427$, $P = .0014$, $n = 52$). A correlation was not observed between IFN-γ production and viral load measured at death. These data demonstrate the importance of humoral immunity against viral encephalitis, as shown in other studies (Levine *et al*, 1991; Ubol *et al*, 1995).

The DNA-prime, oral protein-boost vaccination strategy used in this study was immunogenic and provided partial protection against viral encephalitis. Several aspects of the prime-boost vaccination regimen could be modified to improve the protection afforded against challenge. It is likely that a modified boosting antigen would lead to greater protection against challenge, as the boosting antigen used here may have lacked immunodominant epitopes present on the E2 proteins produced following DNA vaccination. The use of another expression system to produce this protein, such as plants, yeast, and mammalian cell culture, may result in an improved vaccine antigen.

Age-dependent resistance to SV-induced encephalitis in mice is well established (Labrada *et al*, 2002). Therefore, the murine model of SV-induced encephalitis requires the use of a relatively 'compressed' vaccination schedule to ensure that mice are susceptible to encephalitis when they are challenged. As the time interval between vaccine doses required to maximize immunity is generally considerably longer, particularly in prime-boost vaccination regimens, it is likely that substantial improvements in protective immunity can be obtained by optimizing
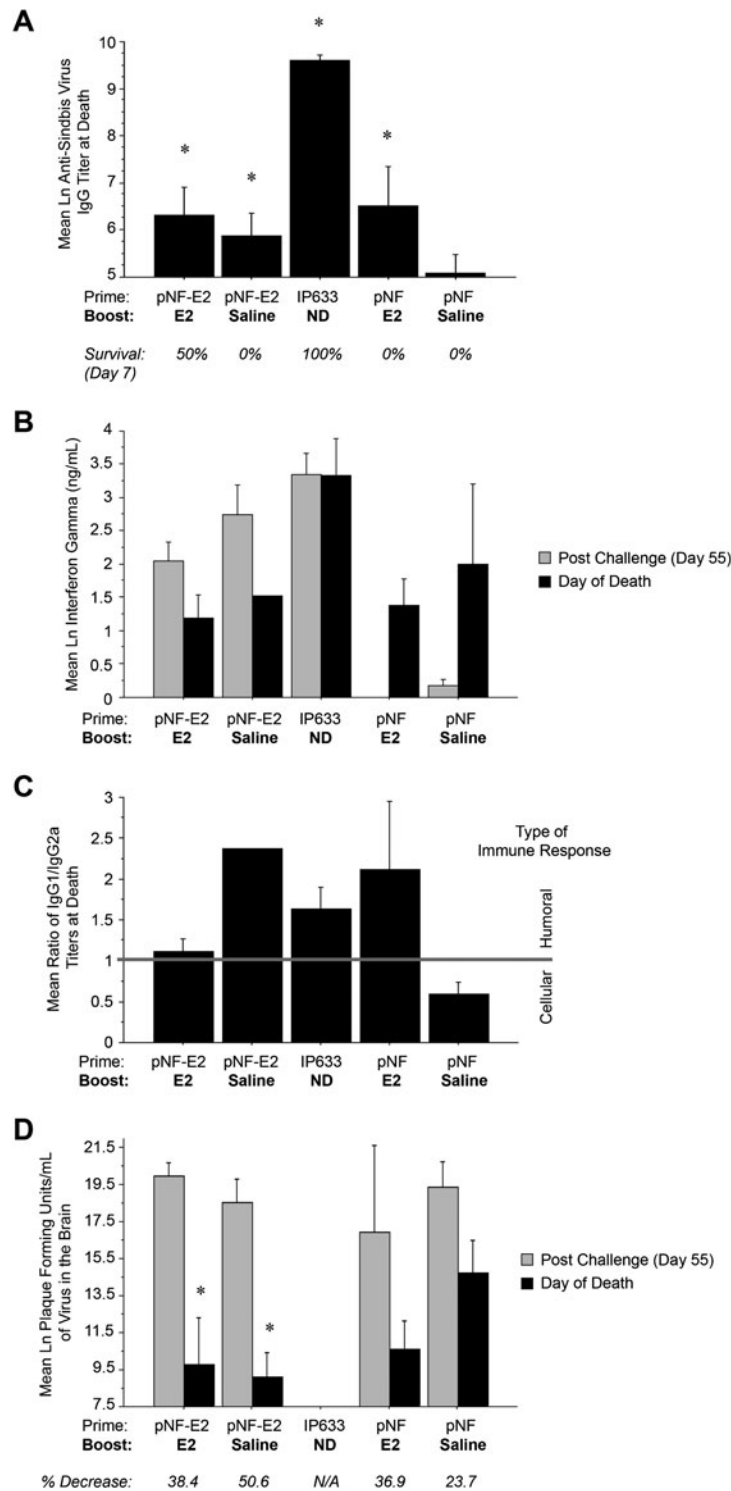
Prime-boost vaccination against viral encephalitis
MP Zanin *et al*
287

Figure 3 Immune responses and viral load measured at day 55 and at death following vaccination using prime-boost vaccination regimens. Data are log$_e$ transformed and mean values for each group are shown, with error bars indicating standard error. Mice were injected with DNA on days 0 and 14 and administered protein boosts on days 21, 28, and 35. Mice were challenged on day 49. (**A**) Anti-Sindbis virus IgG titers measured by ELISA using serum samples obtained at death ($n = 6$). Percentage survival of these groups measured on day 56 (7 days post challenge) is also shown. *Statistically significant difference compared to the pNF-saline group ($P < .02$). (**B**) Cellular immune responses were measured by stimulating splenocytes with ultraviolet light–inactivated Sindbis virus strain 633 and measuring IFN-γ production by ELISA. Splenocytes were isolated post challenge on day 55 ($n = 4$) and at death ($n = 6$). Data from the pNF-E2 group was not obtained on day 55. (**C**) Anti-Sindbis virus IgG1 and IgG2a were measured by ELISA using serum samples obtained at death ($n = 6$). (**D**) Viral loads were measured via plaque assay on homogenized brain tissue using baby hamster kidney cell monolayers. Brain tissue was isolated post challenge on day 55 ($n = 4$) and at death ($n = 6$). Virus was not detected in mice in the IP633 group. Percentage differences between viral loads measured 5 days post challenge and at death in each group are also shown. *Statistically significant difference compared to the pNF-saline group in the day of death data set ($P < .05$). ND, not done.

the vaccine schedule. Although the 'compressed' vaccination schedule is a limitation of the murine model used in this study, it does suggest that the data presented here may be an underestimate of the potential efficacy of these vaccines.

Collectively, the immunological data presented here demonstrate the importance of humoral immunity against viral encephalitis. They also indicate that cellular immunity against viral encephalitis may be detrimental, as observed when comparing the cellular immune response measured in unvaccinated mice to that measured in vaccinated mice at death and the IgG1/IgG2a ratios observed in vaccinated and unvaccinated mice at death. Published data concerning the role of cellular immunity highlight both positive and negative impacts on the affected animal (Binder and Griffin, 2001; Rowell and Griffin, 2002). The data obtained in this study suggest that, overall, cellular immunity is probably not desirable and may impede recovery from encephalitis. The vaccination strategy used in this study has been shown to skew the immune response towards humoral immunity, which has been shown to be protective against viral encephalitis in the absence of cellular immunity (Levine *et al*, 1991). Considering the potentially negative impact of a cellular immune response, the potential of this vaccination strategy is evident.

This study lends weight to the concept of using oral vaccination against a viral infection of the central nervous system and that, with further development, this strategy could prove its applicability in the management of endemic encephalitis, especially in resource poor regions. Similar results have also been shown in other studies using prime boost strategies and oral vaccines against viral encephalitis (Perkins *et al*, 2006; Ramakrishna *et al*, 1999). Such a vaccine strategy has the potential to solve many of the issues associated with vaccines currently in use against viral encephalitis, thus greatly increasing vaccine coverage. This would have a major impact on both traveller's health and, more importantly, health care in resource-poor regions.

References

Berg SW, Mitchell BS, Hanson RK, Olafson RP, Williams RP, Tueller JE, Burton RJ, Novak DM, Tsai TF, Wignall FS (1997). Systemic reactions in U.S. Marine Corps personnel who received Japanese encephalitis vaccine. *Clin Infect Dis* **24:** 265–266.

Binder GK, Griffin DE (2001). Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons. *Science* **293:** 303–306.

Chen HW, Pan CH, Liau MY, Jou R, Tsai CJ, Wu HJ, Lin YL, Tao MH (1999). Screening of protective antigens of Japanese encephalitis virus by DNA immunization: a comparative study with conventional viral vaccines. *J Virol* **73:** 10137–10145.

Deliyannis G, Boyle JS, Brady JL, Brown LE, Lew AM (2000). A fusion DNA vaccine that targets antigen-presenting cells increases protection from viral challenge. *Proc Natl Acad Sci USA* **97:** 6676–6680.

Dropulic LK, Hardwick JM, Griffin DE (1997). A single amino acid change in the E2 glycoprotein of Sindbis virus confers neurovirulence by altering an early step of virus replication. *J Virol* **71:** 6100–6105.

Griffin DE (1981). Immunoglobulins in the cerebrospinal fluid: changes during acute viral encephalitis in mice. *J Immunol* **126:** 27–31.

Griffin DE, Johnson RT (1977). Role of the immune response in recovery from Sindbis virus encephalitis in mice. *J Immunol* **118:** 1070–1075.

Hanna JN, Ritchie SA, Phillips DA, Lee JM, Hills SL, van den Hurk AF, Pyke AT, Johansen CA, Mackenzie JS (1999). Japanese encephalitis in north Queensland, Australia, 1998. *Med J Aust* **170:** 533–536.

Hanna JN, Ritchie SA, Phillips DA, Shield J, Bailey MC, Mackenzie JS, Poidinger M, McCall BJ, Mills PJ (1996). An outbreak of Japanese encephalitis in the Torres Strait, Australia, 1995. *Med J Aust* **165:** 256–260.

Igarashi A, Tanaka M, Morita K, Takasu T, Ahmed A, Akram DS, Waqar MA (1994). Detection of west Nile and Japanese encephalitis viral genome sequences in cerebrospinal fluid from acute encephalitis cases in Karachi, Pakistan. *Microbiol Immunol* **38:** 827–830.

Jan JT, Griffin DE (1999). Induction of apoptosis by Sindbis virus occurs at cell entry and does not require virus replication. *J Virol* **73:** 10296–10302.

Labrada L, Liang XH, Zheng W, Johnston C, Levine B (2002). Age-dependent resistance to lethal alphavirus encephalitis in mice: analysis of gene expression in the central nervous system and identification of a novel interferon-inducible protective gene, mouse ISG12. *J Virol* **76:** 11688–11703.

Levine B, Hardwick JM, Trapp BD, Crawford TO, Bollinger RC, Griffin DE (1991). Antibody-mediated clearance of alphavirus infection from neurons. *Science* **254:** 856–860.

Maharaj I, Froh KJ, Campbell JB (1986). Immune responses of mice to inactivated rabies vaccine administered orally: potentiation by *Quillaja* saponin. *Can J Microbiol* **32:** 414–420.

Nargi-Aizenman JL, Griffin DE (2001). Sindbis virus-induced neuronal death is both necrotic and apoptotic and is ameliorated by *N*-methyl-D-aspartate receptor antagonists. *J Virol* **75:** 7114–7121.

Perkins SD, O'Brien LM, Phillpotts RJ (2006). Boosting with an adenovirus-based vaccine improves protective efficacy against Venezuelan equine encephalitis virus following DNA vaccination. *Vaccine* **24:** 3440–3445.

Pickering RJ, Smith SD, Strugnell RA, Wesselingh SL, Webster DE (2006). Crude saponins improve the immune response to an oral plant-made measles vaccine. *Vaccine* **24:** 144–150.

Ramakrishna C, Desai A, Shankar SK, Chandramuki A, Ravi V (1999). Oral immunisation of mice with live Japanese encephalitis virus induces a protective immune response. *Vaccine* **17:** 3102–3108.

Rowell JF, Griffin DE (2002). Contribution of T cells to mortality in neurovirulent Sindbis virus encephalomyelitis. *J Neuroimmunol* **127:** 106–114.

Schoepp RJ, Johnston RE (1993). Directed mutagenesis of a Sindbis virus pathogenesis site. *Virology* **193:** 149–159.

Tsai TF, Chang GJ, Yu YX (1999). Japanese Encephalitis Vaccines. In: *Vaccines*. Plotkin SA, Orenstein WA (eds). Philadelphia: WB Saunders, pp 672–710.

Ubol S, Levine B, Lee SH, Greenspan NS, Griffin DE (1995). Roles of immunoglobulin valency and the heavy-chain constant domain in antibody-mediated downregulation of Sindbis virus replication in persistently infected neurons. *J Virol* **69:** 1990–1993.

WHO (2006). Japanese encephalitis vaccines. *Wkly Epidemiol Rec* **81:** 331–340.

Zheng BJ, Ng MH, Chan KW, Tam S, Woo PC, Ng SP, Yuen KY (2002). A single dose of oral DNA immunization delivered by attenuated *Salmonella typhimurium* down-regulates transgene expression in HBsAg transgenic mice. *Eur J Immunol* **32:** 3294–3304.